Faculty of Resource Science and Technology

Determination of TTX by HPLC of Horseshoe Crab Collected from Sibu Laut, Telaga Air and Pasir Putih, Muara Tebas.

Mohamad Nor Fakihin Aqsa Bin Mohd Nor Azam (31066)

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This dissertation is submitted in partial fulfilment of requirement for the degree of Bachelor Science with Honour in Aquatic Resource Science and Management

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DECLARATION

No portion of the work referred to this desertion has been submitted of an application for another degree of qualification of this or any other university or institution of higher learning.

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Mohamad Nor Fakihin Aqsa Bin Mohd Nor Azam

Aquatic Resource Science and Management

Department of Aquatic Science

Faculty of Resource Science and Technology

University Malaysia Sarawak

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List of Abbreviations

℃	Degree Celcius
Cm	Centimeter
g	Gram
HPLC	High Performance Liquid Chromatography
M	Molar
ml	Milliliter
mm	Millimeter
MU	Mouse Unit
MU/g	Mouse unit per gram
rpm	Rotation per minutes
TLC	Thin Layer Chromatography
TTX	Tetrodotoxin
µl	Microliter

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Aquatic Resource Science and Management
Faculty of Resource Science and Technology
University of Malaysia Sarawak

ABSTRACT

In this study, the 16 sample of horseshoe crab, *T. gigas* and *C. rotundicauda* were collected from the Sibu Laut, Telaga Air and Pasir Putih, Muara Tebas. The morphological measurement (i.e body weight and carapace width) were measured by standard measuring, electronic balance and electronic scale to assess the correlation with the toxicity level. For determination of tetrodotoxin (TTX), the High Performance Liquid Chromatography was used. TTX analysis was performed by using Water 600 Cooler system by using Symmetry C18 5µm (4.6 x 150mm) column. Toxins are separated from the contaminants by a buffer solution on a reversed-phase column packed with C18 resin with an ion-pair reagent (sodium 1-heptanesulfonate; HSA). Toxins were detected by fluorescence detector (Waters 2475 Multi Fluorescence Detector) at 505 nm emission with 381 nm excitation. The sample produce a main peak, with the retention time (Rt) was compared with authentic TTX. TTX results outcome were converted to Mouse Unit (MU) in order to compare to human consumption rate. Toxicity score in the eggs of *T. gigas* ranged from 0.02 MU/g to 0.62 MU/g, which in the tissue from 0.03 MU/g to 1.40 MU/g. Toxicity in the eggs of *C. rotundicauda* ranged from 0.34 MU/g to 2.39 MU/g while in tissue ranged from 0.81 MU/g to 3.21 MU/g. Based on the Pearson Correlation coefficient for *T. gigas* crab is 0.399 and C. rotundicauda is 0.051. The carapace width has weak negative correlation with toxicity level of the horseshoe crab. In this study found that, Pasir Putih has higher toxicity level compared with Sibu Laut. The toxicity ranged of *C. rotundicauda* eggs from Sarawak was much lower compared from the Thailand, Myanmar, Bangladesh, and Vietnam. Sarawak show low level of toxicity which are 0.34-2.39 MU/g compared to the highest level of toxicity from the Vietnam 139.1 MU/g.

Keywords: Horseshoe crabs, Tetrodotoxin (TTX), Pearson Correlation coefficient, High Performance Liquid Chromatography (HPLC)

ABSTRAK

Dalam kajian ini, sebanyak 16 sampel belangkas, T. gigas *dan* C. rotundicauda *telah dikutip dari Sibu Laut, Telaga Air dan Pasir Putih, Muara Tebas. Ukuran morfologikal (cth. Berat badan dan lebar karapas) telah diukur dengan ukuran standard, timbangan elektronik dan skala elektronik untuk mengenalpasti korelasi paras tosik. belangkas. Untuk mengenalpasti tetrodotosin (TTX), Prestasi Tinggi Chromatografi cecair telah digunakan. TTX telah dianalisa dengan menggunakan sistem Water 600 Cooler dengan menggunakan simetri kolum C18 5µm (4.6 x 150mm). Toksin dipisahkan dari bahan cemar oleh penyelesaian penampan pada ruangan dibalikkan-fasa penuh dengan resin C18 dengan reagen ion-pasangan (sodium 1-heptanesulfonate; HSA). Toksin dikesan oleh pengesan pendarfluor (Waters 2475 Multi pendarfluor Pengesan) pada 505 nm dengan pelepasan 381 pengujaan nm. Sampel menghasilkan puncak utama, dengan masa tahanan (RT) telah dibandingkan dengan TTX sahih. Keputusan TTX hasil telah ditukar kepada Unit Tetikus (MU) untuk membandingkan kadar pengambilan manusia. Perincian ketoksikan dalam telur* T. gigas *adalah di antara 0.02 MU / g untuk MU 0.62 / g, yang dalam tisu daripada 0.03 MU / g kepada 1.40 MU / g. Ketoksikan dalam telur* C. rotundicauda *adalah dari 0.34 MU / g untuk MU 2.39 / g manakala dalam tisu adalah di antara 0.81 MU / g kepada 3.21 MU / g. Berdasarkan Pekali Korelasi Pearson,* T. gigas *mencatat 0.399 dan* C. rotundicauda *mencatat 0.051. Lebar karapas mempunyai korelasi yang negative dengan paras toksik. Dalam kajian ini mendapati bahawa, Pasir Putih mempunyai tahap ketoksikan yang lebih tinggi berbanding dengan Sibu Laut. Ketoksikan antara* C. rotundicauda *telur dari Sarawak adalah jauh lebih rendah berbanding dari Thailand, Myanmar, Bangladesh, dan Vietnam. Sarawak menunjukkan tahap rendah ketoksikan yang 0.34-2.39 MU / g berbanding dengan paras tertinggi ketoksikan dari Vietnam 139.1 MU / g.*

Kata kunci: *Belangkas, Tetrodotosin (TTX), Pekali Korelasi Pearson, Prestasi Tinggi Chromatografi cecair*

1.0 Introduction

Horseshoe crabs are not true crabs but chelicerates that live in shallow coastal waters. Horseshoe crab belong to Merostomata Class. This creature also known as "living fossil" because they are almost similar with the species found in the fossil record. This allow them to keep survive in various environmental stresses for the past 150 million years (Kamaruzzaman *et al.,* 2012) The earliest identified species in Canada, live about 445 million years ago which mean before Dinosour age. Among four extant species of horseshoe crab in the world, one species known as *Limulus polyphemus* is distributed at the East coast of North America and *Carcinocorpius rotudicauda*, *Tachypleus giga*, and *Tachypleus tridentatus* is found in the Southeast Asian region. In coastal area of Malaysia, three species have been found and identified as *Carcinocorpius rotudicauda*, *Tachypleus gigas*, and *Tachypleus tridentatus* (Chartterji and Noraznawati, 2009).

Horseshoe crabs are also very important in ecological and economical aspect. In term of ecological aspect, it is apart from being food source of migratory birds (Castro and Myers, 1993). In term of economical aspect, the blue blood in the horseshoe crabs are valuable and high demand. This is due to present of cooper based molecule that used as an endotoxin tester food, drug and pharmaceutical industries. In additions, fisherman have found that horseshoe crabs make excellent bait for conch and eels.

Local people in Malaysia especially in the state of Sarawak do eat horseshoe crab as their food consumption. Public not entirely alert and realise about toxin presence in horseshoe crabs and negative impact to their health. In Malaysia, there were not a lot serious case of food poisoning due its ingestion was reported. There have been several reports of food

poisoning due to consumption of the horseshoe crabs eggs in Thailand (Bovornkitti and Kulkantrakorn, 2004). About 100 people were poisoned resulting of five deaths in 1995 (Kanchanapongkul, 2008). Certain species of horseshoe crab like *C. rotundicauda* is reported to be one of the TTX-bearer. This species was found in Thailand, Bangladesh and Cambodia and it has been reported as toxic (Kungsuwun, *et al*., 1987). Sometime, limited knowledge to differentiate between *T. gigas* and *C. rotundicauda* are main causes in food poisoning cases in Thailand (Miyazawa and Noguchi, 2001).

The causative species and their toxins in Thailand, Bangladesh, Cambodia and Vietnam has already been characterised as TTX (Kungsuwun, *et al*., 1987; Tanu and Noguchi, 1999; Ngy *et al.,* 2007; Dao *et al.,* 2009). TTX is a major toxin in the eggs (Kungsuwun *et al*., 1987; Kanchanapongkul, 2008). Its have extremely potent neurotoxin which can cause death with no effective antidote been found yet.

There has very limited data on toxicity of horseshoe crab in coastal water of Sarawak. This study potentially can create awareness to local people. This study was conducted to identify the toxicity of horseshoe crab at Sibu Laut, Telaga Air and Pasir Putih, Muara Tebas of Sarawak. The obtained result could be useful to local people, government and general public in order to prevent food poisoning due to horseshoe crab consumption.

Therefore, this study aims to: 1) To identify the toxin properties of horseshoe crab by using High Performance Liquid Chromatography (HPLC) method; 2) Determine the toxicity level in horseshoe crab tissues and eggs using HPLC, 3) Determine the correlation between morphometric characteristic and level of toxicity and 4) Compare the toxicity level in Sibu Laut, Telaga Air and Pasir Putih, Muara Tebas.

2.0 Literature review

2.1 Horseshoe Crab

Horseshoe crabs belong to the phylum of Arthropods, which consists of animals having an articulated body and limbs. The three major classes of Arthropods are Insects, Arachnids and Crustaceans (Botton and Loveland, 1992). The horseshoes crab belongs to its own class called Merostomata, which means "legs attached to the mouth." Though they are called "crabs," a quick look at their taxonomy shows that they're not. Normally horseshoes crab will appear on the beach and mangrove area. The horseshoes crab belongs important element of coastal food chain because it provides an important food source for migratory birds and sea turtles (Castro and Myers, 1993). This animal is very valuable and in high demand throughout the world as the horseshoe crab blue blood can be used as a serum or assay to detect bacteria and toxins (Akbar John *et al*., 2012).

The horseshoes crab belong in benthic communities and prefer the calm sea or estuary and sandy and muddy areas for their biogenic activity (Nur Izzatie and Samsur, 2011). The main food for these organisms is shellfish or crustacean. However, they also eat a variety of benthic organisms including annelids, and polychaete worms (Carmichael *et al*., 2003). During full and new moon season, the spawning activities of horseshoe crabs is height (Shuster and Botton, 1985; Jennifer *et al*., 2010). Horseshoe crab have to migrate from deeper water to shore specifically for breeding proposed.

The horseshoe crab fertilization process is occurs externally (Hajeb *et al*., 2009). Horseshoe crabs lays their egg at the beach. The females deposited their eggs while males then fertilize it. Based on study by Chatterji and Shaharom (2008), the grain size of sediment are

important factor when involve in the selection of nesting sites of horshoes crab. High salinity can induce the spawning activities. The egg masses were buried at sediment depths of approximately 7-20 cm (Botton *et al*., 1996; Jennifer *et al*., 2010). The complete life cycle of horseshoe crab are shown in figure 1.

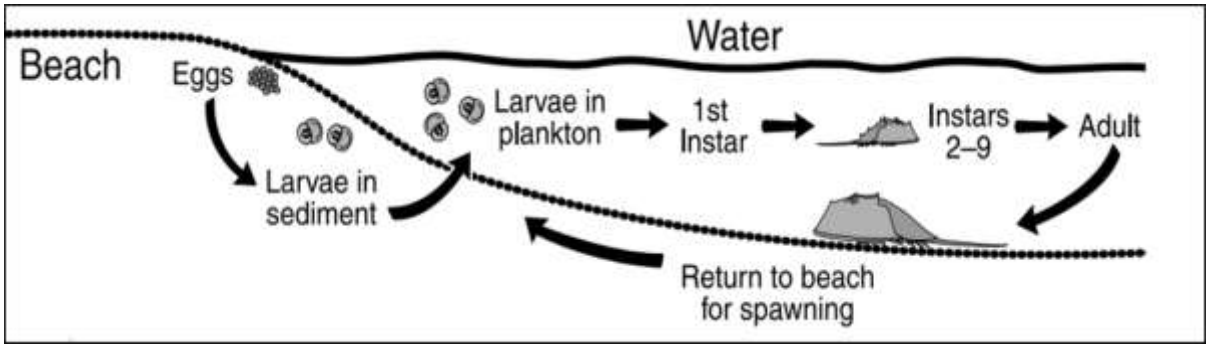


Figure 1: Horseshoe crab life cycle

The distribution of horseshoe crab can be found worldwide. *L. polyphemus,* the American horseshoe crab can be found on Atlantic coast of North America. *T. tridentatus* on the northen shores of Japan up to south Vietnam, *T. gigas* on the shore of Bay Bengal, particulaly along the coast of India to Indo China, and *C. rotundicauda* on northern shores of the Bay of Bengal to the southern coast of Philippines (Chatterji and Abidi, 1993).

Three of them can be found on the coast of Malaysia and are identified as *C. rotundicauda, T. gigas* and *T. tridendatus* (Chartterji and Noraznawati, 2009). Three species of them *C. rotundicauda, T. gigas,* and *T. tridentatus* often eat by people especially in Borneo. *T. gigas* also known as coastal horseshoe crab are mainly found in coastal habitat. Its have been found in most all coastal state including East and West Malaysia. While *C. rotundicauda* also known as mangrove horseshoe crab or "belangkas ranggar or belangkas padi" in Sarawak can be found in mangrove area (Nur Izzatie and Samsur, 2011). They only can be found at

several states, Johor, Terengganu, Selangor, Sabah, and Sarawak. In Sabah, only one species of horshoeshoe crab can be found in coastal area such as *T. tridentatus.*

2.2 Morphology Characteristic of Horseshoe Crabs

The general characteristics is shown in figure 2, the horseshoe crab body is composed of three main part that are the promosa, opithosoma and the telson. In prosoma part, the semicular part of horseshoe crab, consists of a sizeable intestinal tract with an esophagus and proventriculus (nervous system) concentrated into a bulbous brain, a tubular heart, excretory glands at the bases of the walking legs and connective tissue and cartilagenous plates. The opithosoma contains mainly the musculature for the operation of the book gills and a connection to the third part that is the tail or telson (Susan, 2007).

There has some part do distiguish between male and female by observing it size and front pedipalps. Female size is always bigger than male. Large size in females important in order to tow males during the spawning season and a large body can carry more eggs (Botton and Loveland, 1992). In figure 3, the front pedipals of male look like a boxing glove after it reach maturity. While the shape of female horseshoe crab remain the same in which resemble a scissor shape. Male posser a big arms to hold female carapace during mating. In table 1, shows the certain part which can distiguish other species based on morphological characteristic.

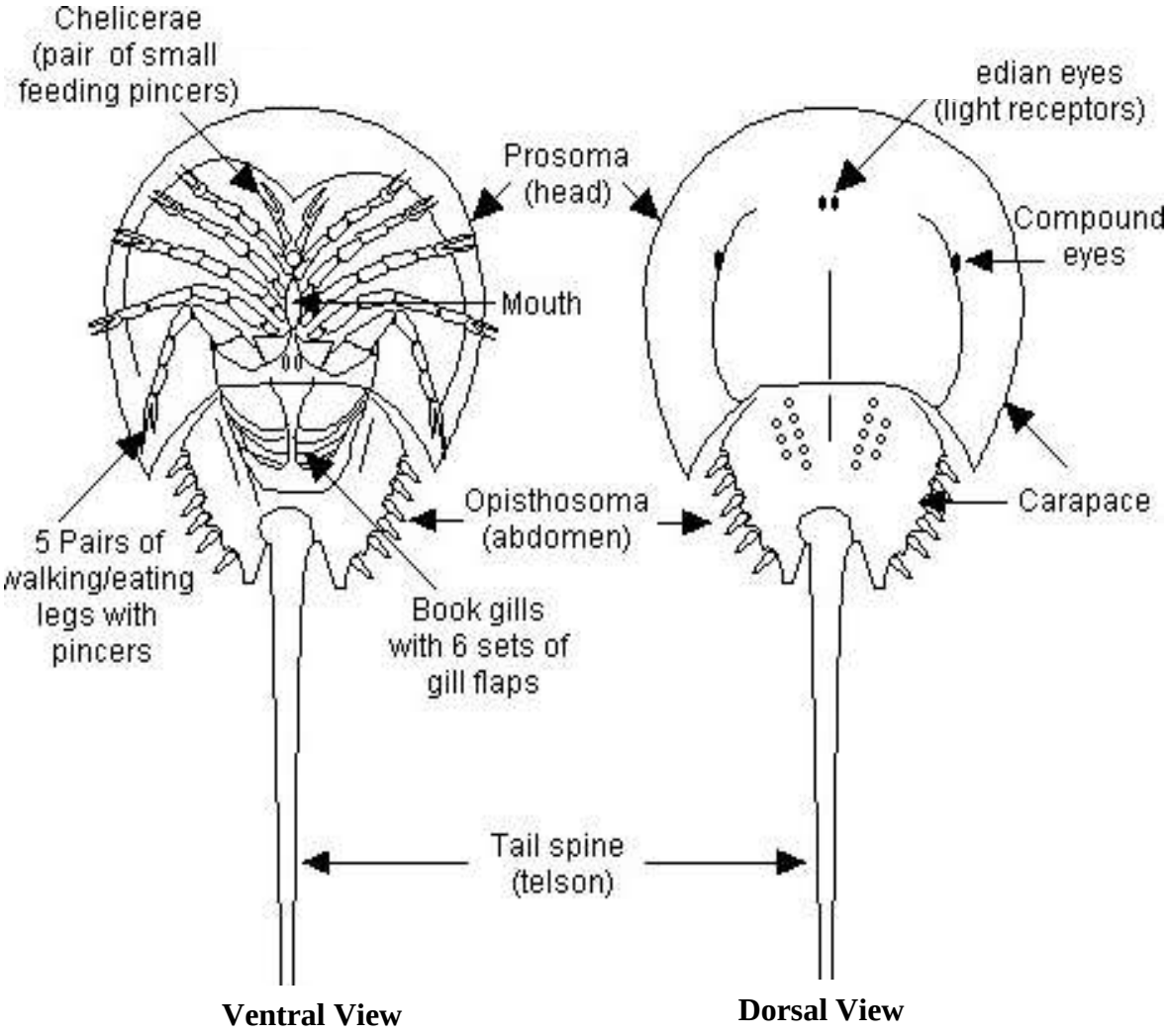
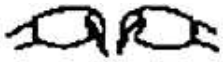


Figure 2: The physical structure of horseshoe crab (McElhatton, 2013)

a b

Figure 3: Structure of the first leg a) female b) male (Gerhart, 2007)

Table 1: Morphological characteristics of the two species of horseshoe crabs (Sekiguchi and Nakamura 1979)

Part	Species	
	Tachypleus gigas	*Carcinoscorpius rotundicauda*
Frontal view		
2nd prosoma appendages		
3rd prosoma appendages		
Telson cross section		
Getinal operculum		
Marginal spines		

2.3 Chemistry of Tetrodotoxin (TTX)

Tetrodotoxin were believe present in the Tetraodontidae family of the puffer fish. It can be found in both terrestrial and marine organism. It is catogerize a non-protein molecule that is soluble in water. TTX is a heat stable toxin in neutral to weakly acidic solutions which means its does not decompose even by cooking (Arakawa *et al*., 2010). TTX is an amino

perhydroquinazoline compound with a molecular formula of $C_{11}H_{17}N_3O_8$ (Hashimoto, 2001). The molecular weight compound of TTX is low, 319.

Based on this TTX structure, TTX derivatives were isolated from puffers, newst, and a frog, which were named as 4-*epi* TTX, 6-*epi* TTX, 11-deoxyTTX, 11-deoxy-4*epi*TTX, 11-*nor*TTX-6(*R*)-ol, 11-*nor*TTX-6(*S)*-ol, 11-*nor*TTX-6,6-diol, 4,9-anhydroTTX, 11-*oxo*TTX, 4,9-anhydro-4-*epi*TTX, 4,9-anhydro-11-deoxyTTX, 5-deoxyTTX and tetrodonic acid (Miyazawa and Noguchi, 2001)

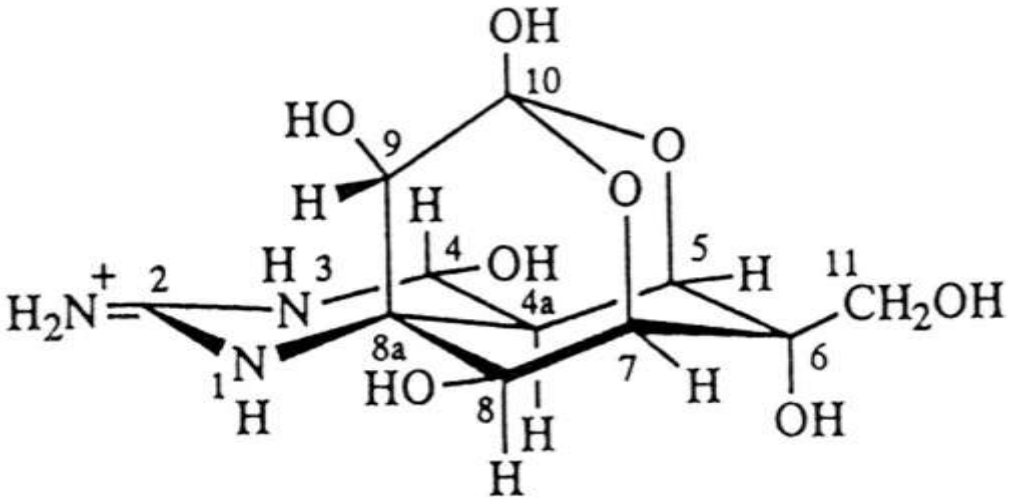


Figure 4: The structure of TTX (Tanu and Noguchi, 1999)

2.4 Symptoms of TTX poisoning

There are a lot of cases poisoning due to TTX. Usually, the symptons will appear with 10-45 minutes of exposure, but some cases reported that it can reach as 3 to 6 hours after exposure by depending the amount of toxin ingested (Tanu, 2000). There has several stages

that show the sympton of intoxification. Firstly, slight numbness of the lips and tongue. Next, the sensations of lightness or floating, headache, diarrhea may occur (Tanu, 2000).

The second symptom caused by TTX usually is paralysis. In severe cases respiratory paralysis occurred and lead to death. The type, severity and variety of symptoms depend on the amount of toxin ingested, age and health of the victim (Noguchi and Ebesu, 2001). TTX will blocks sodium channel on the cell membrane and inhibits sodium ion flow in and out of the cell, resulting in paralysis of the muscle concerned (Hashimoto, 2001).

TTX can act on both the central and peripheral nervous system. Sensory neurons are affected first and then motor neurons at a higher dose of TTX (Wan *et al.,* 2007). There is no effective antidote or specific treatment for TTX to eliminate the toxin from the human body. The victim only can be help using artificial respiration treatment. This treatment only slowdown the death process without any significant treatment.

2.5 Treatment

Since no antidotes for TTX poisoning, treatment is mainly supporting therapy, mechanical ventilation for oxygen supply, normal saline infusion, gastric emptying procedure, treatment with dopamine and normal saline infusion for distending intravascular volume (Noguchi & Arakawa, 2008). According to Tanu (2000) stated that, to reduce the exposure for unabsorbed TTX, emetics may be admisnistered if the vomiting does not already appeared. Fluid loss can be undergoes of therapy such as fluid and electrolyte replacement therapy (Wan *et al,.* 2007)

2.6 Toxification mechanisme of TTX in marine organism

The main origin of toxin in marine organism can be either endogenous or exogenous. Organism that produced their own toxin called endogenous without influenced by the other organism while organism get toxin sources from the outside are known as exegenous and influenced by the other organism. According to Tanu *et al.* (1999) proved that the main sources of TTX is not come from the crab itself but it is from the prey that the crab ate. Diet feeding for horseshoe crab are mollusk, arthropods and detritus that may contain TTX carrying bacteria (Tanu *et al.*, 1999).

Studied done by Noguchi and Arakawa (2008) proved that major way how the TTX is accumulated in the marine animals was derived from the food chain. In their experiment showed that puffer fish that has been culture with the non TTX diet were non-toxic. The puffer fish become toxic when it supplied with the food that contain TTX. In figure 6 was illustrated the pathway of accumulation of TTX in marine organism via the food chain.

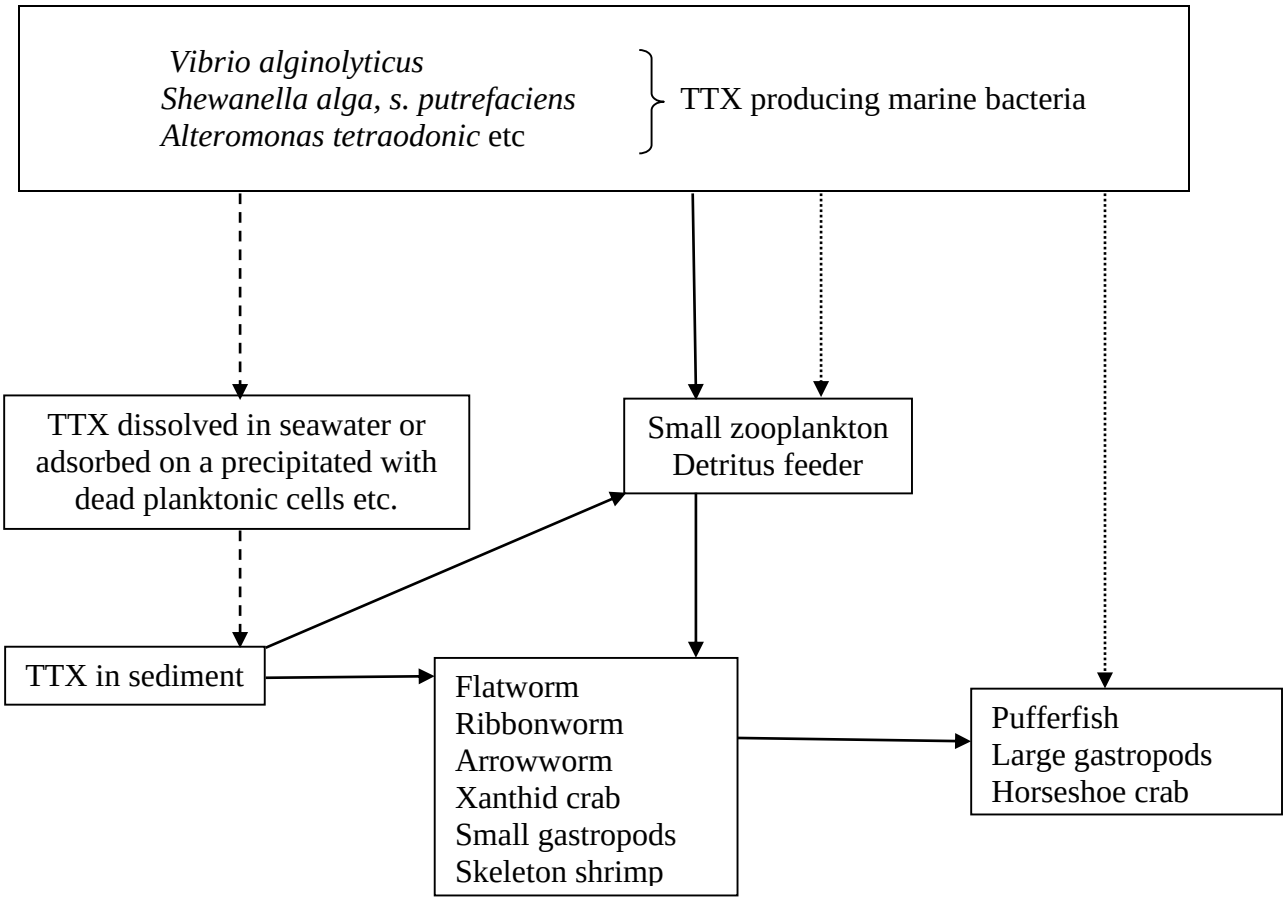


Figure 5: Illustration of mechanism of TTX accumulation (Edited from: Noguchi & Arakawa, 2008)

There has other ways the accumulation of TTX in marine organism can occur via direct interaction with the TTX producing bacteria. According to the Moczydlowski (2013), there has more than 12 different species of TTX producing bacteria found in the puffer fish. *Vibrio spp* are the main bacteria that produce TTX in the horseshoe crab (Kungsuwun, *et al*., 1987). *Vibrio spp* was react as the parasite and do symbion directly with marine organism, then accumulated inside their body without via food chain. The contribution of TTX produce through this mechanism is small amount (Noguchi and Arakawa, 2008).

2.7 Poisoning Case Due to Consumption of Horseshoe Crab

Many cases reported due to human ingestion of puffer fish. In Malaysia, there were little seriuos cases of poisoning cases information due to ingestion of horseshoe's crab eggs being reported. There has several cases that victim are refuse to go to the hospital. Hence, no proper clinical data be recorded.

Poisoning case due to consumption of horseshoe crab eggs has sprodically report in Asian region. According to Kungsuwun *et al.,* (1987), in Thailand since 1925, there has many cases occurred due to ingesting of horseshoe crab especially their eggs, and about 100 people were poisoned in April 1995. Thailand's people do eat on horseshoe crab as their meal. There are many symptom have detected to the victims who got horseshoe crab poisoning. Within several hours after ingestion dizziness, nausea, headache, numbness of lips, aphasia, and paralysis of limbs appear. In serious case patient died within 5 hours.

A study by Kungsuwun *et al.,* (1987), reveal that from all the three species, *C. rotundicauda* is the toxin species, while *T. gigas* and *T. tridentatus* are non toxic. Usually, *T. gigas* and *T.*

tridentatus are consumed by people but sometimes they mistaken it with *C. rotundicauda* because it look like the same. Within 1994 to 1996, medical service of Chon Buri Hospital admitted 280 cases of poisoning case due to consumption of toxic eggs of the *C. rotundicauda.*

In 1999, the study by Tanu and Noguchi shows that, horseshoe crab, *Carcinoscorpius rotundicauda* in Bangladesh also has high level of tetradotoxinc. In Cambodia, the toxicity studies in horseshoe crab was continued. Combodian horseshoe crab, *C. rotundicauda* also contain high toxicity level and not suitable to eat. There has several poisoning cases occurred due to ingestion of horseshoe crab in fisherman community (Ngy *et al.*, 2007)

In coastal areas of South Vietnam, got food poisoning, including fatal cases caused by ingestion of horseshoe crab *C. rotundicauda*. Recent study by Dao *et al* (2009), displayed that occurrence of toxic specimens of *C. rotundicauda* in Vietnam is tremendously high. All the samples collected are toxic and show high toxicity level which is (50.3 to 81.2 MU/g). these toxicy levels are much higher that safe consumption level of TTX (10 MU/g) suggested in Japan.

2.5 TTX assessment

TTX detections is widely studied by many reseacher for assessment of level toxicity various organisms such as puffer fish, horseshoe crab and others. There has several method that commonly used such as mouse bioassay, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), H-NMR spectrum and Ultra liquid chromatography-mass spectrometry.

Presently the mouse bioassay still forms the basis of most toxicity level monitoring programmes. Mouse toxicity assay is similar to the method developed for PSP (paralytic shelfish poisoning) toxin monitoring (AOAC, 1995) which has been applied to determine the toxicity level among puffer fish. Mouse unit (MU) is the unit for the lethal potency scale. Based on Kawabata (1978), only 0.22µg amount of TTX can caused death, in 30 min, a male mouse of ddY strain weighing 20 g was estimated as one mouse unit (MU). Mouse bioassay show low sensitive and precision and its also required a continous supply of mice of a constant size.

Furthermore, the toxin composition detail was not shown in the mouse assay assessment. According to Arakawa *et al.* (2012), the limition of mouse bioassay are cannot distinguish TTX from other neurotoxin such as paralytic shellfish poison (PSP). Chromatographic technique like thin layer chromatography (TLC) is a used to separate the components of a mixture using a thin stationary phase supported by an inert backing. TLC is an analytical tool and it was widely used due to its simplicity, relative low cost and speed of seperation.